

microorganism under conditions suitable for expression of said secretion factor and secretion of said protein.

In one embodiment, the host cell is transformed with a first DNA sequence encoding a signal peptide operably linked to a second DNA
5 sequence encoding a protein. Said protein may be, but not limited to, hormones, enzymes, growth factors, cytokines, antibodies and the like. In another embodiment, the enzyme includes, but is not limited to hydrolases, such as protease, esterase, lipase, phenol oxidase, permease, amylase, pullulanase, cellulase, glucose isomerase, laccase and protein disulfide
10 isomerase. The second DNA sequence may encode a protein that has been modified such that its carboxy-terminus possesses at least one, preferably two, charged amino acids. Such modification may be by substitution of the native carboxy-terminal residues or addition of a tag sequence to the native protein's carboxy-terminus.

Further provided herein is a method of enhancing resistance to
15 proteolysis of a protein. In a preferred embodiment the protein is a secreted protein. It is contemplated that the protein will comprise a tag wherein the tag comprises at least one charged amino acid residue. The charged amino acid residue may be either a positively charged residue or it may be a negatively
20 charged residue.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of
25 illustration only, since various changes and modifications within the scope and spirit of the invention will become apparent to one skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. A. Northern blot of total RNA of *B. subtilis* 168 and *B. subtilis*
30 168 Δ ssrA, hybridized with an ssrA specific probe. At the bottom: the level of

16S RNA in both RNA samples. B. Growth curves of *B. subtilis* 168 (---o---) and *B. subtilis* 168 Δ ssrA (—●—) at 37 °C in TSB medium. C. Growth of *B. subtilis* 168 and *B. subtilis* 168 Δ ssrA on HI-agar plates at 25 °C or 45 °C.

Figure 2. hIL-3 expressed from an mRNA without a stop codon

5 (pLATIL3TERM), accumulates in the medium of *B. subtilis* lacking SsrA (lanes 3, 6, 9), but not in cells containing functional SsrA (lanes 2, 5, 8). At three different growth stages, samples were collected from cultures of *B. subtilis* 168 (pLATIL3) [lanes 1, 4, 7], *B. subtilis* 168 (pLATIL3TERM) [lanes 2, 5, 8], and *B. subtilis* 168 Δ ssrA (pLATIL3TERM) [lane 3, 6, 9]. After
10 centrifugation, the proteins in the culture supernatants were concentrated by TCA precipitation and analyzed by SDS-PAGE and Western blotting with anti-hIL-3 antibodies. The amount of total extracellular protein of *B. subtilis* 168 (pLATIL3) that was applied to the gel [lanes 1, 4, 7] was 10 times less than that of *B. subtilis* 168 (pLATIL3TERM) [lanes 2, 5, 8] or *B. subtilis* 168 Δ ssrA
15 (pLATIL3TERM) [lanes 3, 6, 9]. M indicates a lane with a prestained protein ladder; the molecular weight of the upper band corresponds to 20 kDa, that of the lower band to 15 kDa.

Figure 3. Stability of hIL-3 variants with different C-terminal tags.

(A). Western blot analysis of hIL-3 protein variants produced by *B. subtilis* 168 transformed with plasmid pLATIL3 (lane 1), pLATIL3BStag
20 (expression of hIL-3 with a C-terminal *B. subtilis* SsrA tag (AA-tag): hIL-3-AGKTNSFNQNVALAA; lane 2), pLATIL3DDtag (expression of hIL-3 with a DD-tag: hIL-3-AGKTNSFNQNVALDD; lane 3), and pLATIL3ECtag (expression of hIL-3 with a C-terminal *E. coli* SsrA tag (EC-tag): hIL-3-
25 AANDENYALAA; lane 4). Culture supernatants of cells entering the stationary phase were collected and analyzed by SDS-PAGE and Western blotting with anti-hIL-3 antibody.

(B). Pulse-chase assays: Cells of *B. subtilis* 168 (pLATIL3BStag) and 168 (pLATIL3DDtag) were labeled with [³⁵S]-methionine for 1' prior to chase
30 with excess non-radioactive methionine. Samples were withdrawn at the

times indicated, centrifuged and the culture supernatants were analyzed by SDS-PAGE and fluorography.

(C). The amounts of hIL-3-AAtag and hIL3-DDtag in (B) were quantified by determination of the radioactivity in the dried gel using a PhosphorImager (Molecular Dynamics) and plotted.

Figure 4. The 'major extracellular proteases' of *B. subtilis* play a role in the degradation of extracellular, SsrA-tagged h-IL3. Western blot analysis of hIL-3 protein secreted by *B. subtilis* 168 harboring plasmid pLATIL3 (lane 1, 6) or pLATIL3TERM (lane 2, 7), and *B. subtilis* WB600 (a multiple protease negative strain) containing plasmid pLATIL3TERM and expressing either wild-type SsrA (lane 3, 8), no SsrA (lane 4, 9) or SsrA^{DD} (lane 5, 10). Culture supernatants of cells entering the stationary phase were collected, concentrated by TCA precipitation, analyzed by SDS-PAGE and immunoblotting with anti-hIL-3 antibody (lanes 1-5) or anti-Bs-SsrAtag antibody (lanes 6-10). SsrA-tagged hIL-3 (lanes 3, 5, 8, 10), run-off hIL-3 translation product (lane 4, and possibly also in lane 3 and 5, see text), and wild-type hIL-3 (lane 1) are indicated by the arrows (→). Protein bands with lower molecular weight that also react with anti-hIL-3 antibody are supposedly degradation products of hIL-3, SsrA-tagged hIL-3 or run-off hIL-3 translation product.

Figure 5. *B. subtilis* CtpA has an additional role in the degradation of SsrA-tagged hIL-3. Western blot analysis of hIL-3 protein secreted by *B. subtilis* WB600 harboring plasmid (pLATIL3TERM) and carrying either no additional mutation (lane 1, 6), or lacking CtpA (lane 2, 7), YvjB (lane 3, 8), ClpP (lane 4, 9), or SsrA (lane 5, 10). Culture supernatants of cells entering the stationary phase were collected, concentrated by TCA precipitation, analyzed by SDS-PAGE and Western blotting with anti-hIL-3 antibody (lane 1-5) or anti-Bs-SsrAtag antibody (lane 6-10). The straight arrows (→) mark SsrA-tagged hIL-3 (lanes 1-4 and lanes 6-9), and run-off translation product (lane 5 and possibly (see text) also in lanes 1-4). Degradation products of